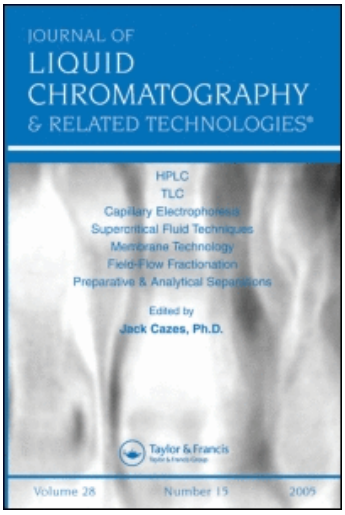

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Yu, Z. and He, Z.(1998) 'Effect of Electrostatic Interaction on Retention Behavior of Proteins in HPSEC', Journal of Liquid Chromatography & Related Technologies, 21: 11, 1579 — 1589
To link to this Article: DOI: 10.1080/10826079808001246
URL: http://dx.doi.org/10.1080/10826079808001246

J. LIQ. CHROM. & REL. TECHNOL., 21(11), 1579–1589 (1998)

EFFECT OF ELECTROSTATIC INTERACTION ON RETENTION BEHAVIOR OF PROTEINS IN HPSEC

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ABSTRACT

The retention behavior of nine proteins was experimentally detected by using high performance size exclusion chromatography (SEC). At low ionic strength of mobile phase, the retention behavior of proteins is dependent of not only size exclusion but also electrostatic interaction. At the same pH value of mobile phase as the isoelectric point of a protein, the protein molecule is neutral. When the pH value of mobile phase is higher than the isoelectric point of a protein, the protein molecule carries a negative charge, resulting in size exclusion and ionic exclusion occuring together. Otherwise, ionic adsorption dominates in electrostatic interaction. At middle ionic strength of mobile phase, the retention behavior is governed by size exclusion mechanism. The experimental result indicated that in order to achieve ideal size exclusion the elution conditions for proteins should be ionic strength 0.1~0.3 M and pH value near its isoelectric point. A linear "calibration curve" was correlated by eight proteins except hemoglobin, in which the related coefficient was as high as 0.9977.

INTRODUCTION

High performance size exclusion chromatography (SEC) is a chromatographic process to separate solutes based on differential permeation in a column packed with porous particles. When proteins ranging in molecular weight from several thousands to several millions (dalton) are swept through such a column, the logarithm of solute's molecular weight is inversely proportional to its elution volume between the limits of total exclusion and total permeation.[1] The ideal size exclusion is only decided by the accessibility of permeate solute molecules into the porous particles and the mass transfer rate in the pores. The retention behavior of solutes is not affected by nonsize exclusion interactions between solutes and the stationary phase.

In SEC, the distribution coefficient of solute is defined by

$$K_d = \frac{V_e - V_0}{V_i} \tag{1}$$

where V_0 is the void volume (may be measured by blue dextran), V_i the pore volume of column support (may be measured by sodium azide) and V_e the elution volume of a solute. In an ideal size exclusion, the range of K_d should be between 0 (total exclusion) and 1 (total permeation). If $K_d > 1$, it is possible that there are nonsize effects.[2]

In addition to size exclusion, there simultaneously are several other factors to affect the retention mechanism in a nonideal SEC: electrostatic interaction, hydrophobic interaction, hydrogen bending, and bioaffinity etc.[3-9]

Electrostatic interaction may occur between protein molecules and packing materials, since most of chromatographic packings have surface with anionic groups, which act as cation exchange sites.[5] Electrostatic interaction mainly includes ion exchange and ion exclusion, which are caused by the presence of dissociated silanol groups on the surface of packing. Cationic protein molecules are adsorbed by ion exchange, and anionic protein molecules are excluded from entering the pores of packing due to electrostatic repulsive forces. As pointed out by Engelhardt, the electrostatic interaction can be reduced or removed by increasing the ionic strength of mobile phase.[3]

Hydrophobic interaction may occur by using aqueous mobile phase of high ionic strength. Due to some hydrophobic groups on packing supports this will induce hydrophobic interaction with nonpolar groups of a protein molecule such as leucine, phenylalanine, tryptophane, alanine, proline, and so on.

Table 1

Porperties of Nine Proteins Used in the Experiments

No.	Protein	Original	Molecular Wt. (Daltons)	Isoelectric Pt.
1	Cytochrome C	Bovine heart	13,370	10.7
2	Lysozyme	Chicken egg white	14,300	11.0
3	β-Lactoglobulin	Bovine milk	35,400	5.2
4	Ovalbumin	Chicken egg	45,000	4.63
5	Hemoglobin	Bovine	64,000	6.7
6	Albumin	Bovine serum	67,000	4.7
7	Conalbumin	Chicken egg white	86,180	5.9
8	γ-Globulin	Horse	149,900	7.1
9	Catalase	Bovine	247,500	6.5

After a systematic study on chromatographic behavior of Sephadex packing, Haglund and Marsden concluded that the hydrophobic interaction can usually be eliminated by reducing ionic strength of mobile phase.[9,10] Hydrogen bending and bioaffinity had been repeatedly noticed in the association of lectins, enzymes, and nucleic acids with various types of soft gel supports. However, the two types of interactions had not been observed with high performance bonded-phase materials.[6]

In order to guide the choice of ideal SEC conditions, the highlight of this work is to identify the effects of nonsize exclusion interactions by experimentally detecting the retention behavior of nine proteins in a SEC column.

MATERIALS AND METHODS

Nine proteins were purchased from Sigma Corp., USA. The physical properties of these proteins were listed in Table 1. Each protein sample was prepared by dissolving in mobile phase of appropriate buffer at pH 6.7 and ionic strength 0.1 M.

High performance SEC was performed using a Waters HPLC consisting of a HPLC pump (Waters 510), a programmable multiwavelength UV detector (Waters 490E), and a computer control system (Waters Maxima 820).

To facilitate the determination of the pore volume, a differential refractometer (Waters 410) was connected in series. The size exclusion column used was the Waters PROTEIN PAK 300 sw (ϕ7.5 x 300mm) modified chemically on the silica based surface. The bonded functional groups should have hydrophilisity, neutrality, and stability. Its exclusion range for protein molecular weight was from 10 kilo-daltons to 400 kilo-daltons.

The buffers of different pH value and ionic strength were used as the mobile phases. Ultrapure water was prepared by Milli-Q Labo (Millipore, Japan). Sodium phosphate and potassium phosphate of analytical grade were added into the water in different proporational rates.

Accounting for the percentage of ionization and multiprotic equilibrium, the pH value and ionic strength were corrected accordingly by the calculations for individual buffer solution. All mobile phases were filtered by 0.2 μm microfiltration membrane and degassed prior to use. The flow rate of mobile phase was 0.6 mL/min in each case.

RESULTS AND DISCUSSION

As mentioned previously, there are several interactions, except size exclusion, to affect the retention behavior of a protein in SEC. Here, we focused on the effect of electrostatic interaction on the distribution coefficient, K_d, in order to guide the choice of the conditions of mobile phase to achieve ideal SEC.

Ionic Strength

Figure 1 showed the experimental results of different ionic strength (from 0.01 to 0.3 M) on the distribution coefficients for six typical proteins at constant pH 6.7. Since either a protein molecule or the surface of porous packing will exhibit different charge natures at different pH and ionic strength of mobile phase, there must be electrostatic attractive or repulsive interaction. As shown in Fig.1, we discuss the effect of ionic strength on K_d for six proteins in three cases.

Cytochrome C and Lysozyme

Because their isoelectric points are over 6.7, the two proteins have net positive charges. In this case, the chromatographic separation reserved a combinative retention mechanism of size exclusion and electrostatic attractive

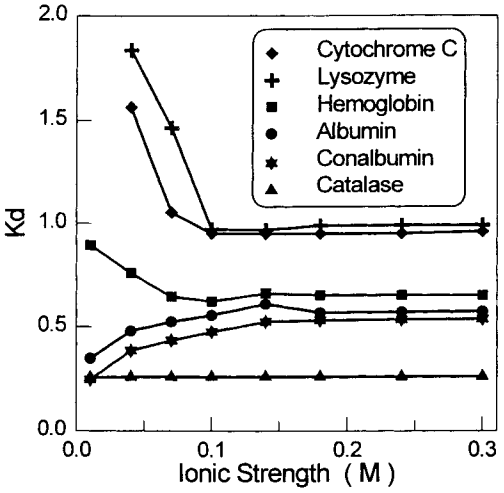

Figure 1. Effect of ionic strength of mobile phase on distribution coefficient at pH 6.7.

interaction. Especially in the region of ionic strength below 0.1 M, the ion adsorption resulted in a rapid increase in distribution coefficient (even over 1.0) because protein molecule with positive charge is more easy to enter into the pores of packing and the retention time is protracted.

Albumin and Conalbumin

The isoelectric points of the two proteins are below 6.7. The electrostatic repulsive force between protein molecules and the surface of porous particles prevents the protein molecules from entering in the pores of packing in the region of low ionic strength. In this case, the retention mechanism includes size exclusion and ionic exclusion. As a result, the latter will cause the distribution coefficient decreased and the retention time curtailed.

Hemoglobin and Catalase

Since the isoelectric points of the two proteins equal to or approach 6.7, the protein molecules should be neutral. Thus, the distribution coefficient of hemoglobin should be, as catalase, not dependent of the pH value of mobile phase. However, the retention mechanism of hemoglobin exhibits a very queer behavior. This might be summarized up the molecular specificity of hemoglobin, and discussed in more detail later.

It must be pointed out that the distribution coefficients of all six proteins were roughly constant when the ionic strength of mobile phase was larger than 0.1 M. This phenomenon could be explained by the fact that the electrostatic interaction, either attractive or repulsive, between protein molecules and the surface of porous packing could be weakened or eliminated by increasing the ionic strength to over 0.1 M.

pH Value

To further explore the effect of pH value on the distribution coefficient, a set of SEC experiments were performed for six proteins at the constant ionic strength 0.1 M and under different pH value. There was a characteristic pH value in the range from 3.5 to 4.0 for the surface silanols of silica-based SEC packings.[5] When increasing acidic condition, the ionization of surface silanols was repressed, therefore, the net charge of the surface decreased.

In contrast, the net negative charge on the surface of silica-based packings would take an important part in a nonideal SEC. As shown in Fig.2, there were three electrostatic interaction modes:

(1) The retention behavior of each protein at pH value below 4.0 could approach ideal SEC owing to ionization of surface silanols on packings.
(2) The distribution coefficients of some proteins such as cytochrome C and lysozyme, at mobile phases pH range from above 4.0 to below their isoelectric points, showed a slowly increasing tendency. This could be explained by the ion adsorption owing to interaction of the opposite charges.
(3) The distribution coefficients of other proteins, such as albumin and conalbumin, if the pH value of mobile phase exceeds the characteristic value of surface silanols and their isoelectric points, showed a slowly decreasing tendency. In this case, ion exclusion played an important part in addition to size exclusion.

An observation of particular interest was that the transition from ion exclusion to ion adsorption occurred at the isoelectric point where the protein charge took a opposite turn. As the pH approached the isoelectric point, the net charge of the protein reduced progressively to so weak that the electrostatic interaction could be eliminated. In this case, the retention behavior of a protein could be regarded as under ideal SEC condition. The transition of hemoglobin had different characterization that its transition point seemed lower than its isoelectric point.

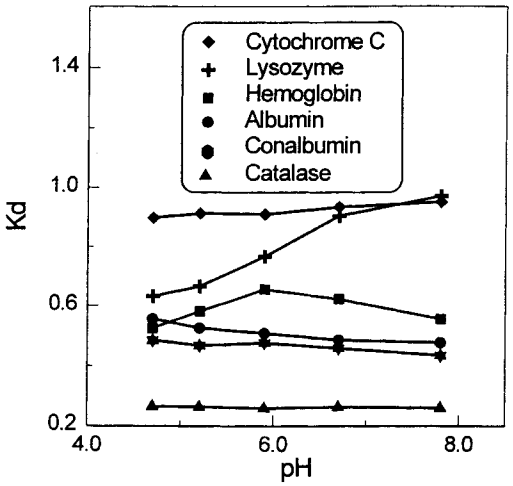

Figure 2. Effect of pH value of mobile phase on distribution coefficient at ionic strength 0.1 M.

Calibration Curve

In ideal SEC, the solute should be separated by size based on different permeation within porous packing. A typical "calibration curve" based on our experimental data in the PROTEIN PAK 300 sw column was shown in Fig.3. The phosphate or acetate buffer was used as the mobile phase at the ionic strength 0.1 M and the pH determined in terms of the isoelectric point of a protein to eliminate nonideal SEC effects.

As shown in Fig.3, the calibration curve showed that there was a typical linear portion of ln M_w vs V_e between the limits of total exclusion (K_d = 0.0) and total permeation (K_d = 1.0).

The correlated equation is:

$$\ln M_w = -0.5632V_e + 16.0416 \qquad (2)$$

where M_w is the molecular weight (dalton) of a protein, V_e the retention volume in the column. The related coefficient of Eq.(2) was 0.9841.

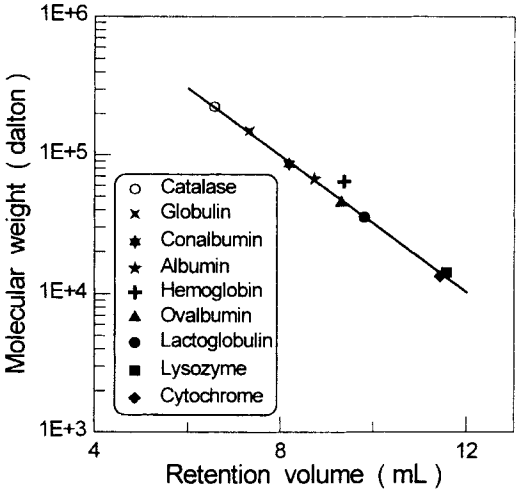

Figure 3. Correlated curve between molecular weight and elution volume.

Retention Behavior of Hemoglobin

As shown in Figs.1~3, we had noticed that the retention behavior of hemoglobin exhibited an obvious divergence from other proteins. To explain this queerity more clearly, we injected a mixed sample consisted of three proteins: albumin, hemoglobin, and ovalbumin into the PROTEIN PAK 300 sw column. The chromatogram was shown in Fig.4. In general, hemoglobin should be the second peak flowing out of the column according to molecular weight sequence. However, the retention time of hemoglobin was unexpectedly longer than that of ovalbumin even through the former molecular weight is far bigger than that of the latter. The reason why hemoglobin exhibited such a large divergence is not yet clear. Perhaps, it might be coming from very strong electrostatic adsorption at low ionic strength even at its isoelectric point. In accordance with the above facts, we strongly suggest that using hemoglobin be avoided for detecting the separation capacity of a SEC column. If hemoglobin was canceled, the new correlation for the "calibration curve" of the PROTEIN PAK 300 sw column becomes:

$$\ln M_w = -0.5664V_e + 160341 \qquad (3)$$

The related coefficient of Eq.(3) was as high as 0.9977. By Eq.(3), the predicted and experimental results of distribution coefficients for nine proteins were summarized in Table 2.

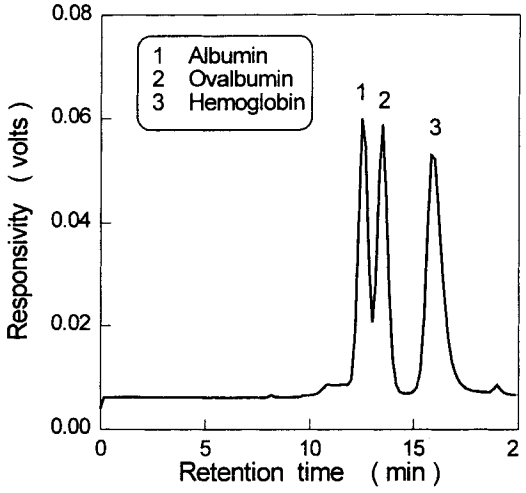

Figure 4. A typical chromatogram in PROTEIN PAK 300 sw column. The injection sample consisted of albumin, hemoglobin and ovalbumin. The amount of solute in sample was 2.0 mg/mL for each protein. The injection volume was 40 μL. The mobile phase was the buffer of a mixture of sodium phosphate and potassium phosphate at ionic strength 0.1 M and pH 6.7, and the elution rate was 0.6 mL/min. The absorbance of each protein was detected at UV wavelength 280 nm.

Table 2

A Comparison Between the Experimental and Predicted K_d by Eq. (3)

Protein	Molecular Wts. (Daltons)	Experimental K_d	Predicted K_d	Relative Error (%)
Cytochrome C	13, 370	0.9507	0.9659	-1.57
Lysozyme	14,300	0.9708	0.9486	2.34
β-Lactoglobulin	35,400	0.7138	0.7151	-0.18
Ovalbumin	45,000	0.6408	0.6533	-1.91
Hemoglobin	64,000	0.6489	0.5626	15.33
Albumin	67,000	0.5559	0.5508	0.93
Conalbumin	86,180	0.4763	0.4860	-2.00
γ-Globulin	149,900	0.3520	0.3434	2.50
Catalase	247,500	0.2419	0.2143	12.88

CONCLUSION

Electrostatic interaction occurs between proteins and charged porous packings at low ionic strength. At ionic strength below 0.1 M, it was shown that electrostatic interaction could be susceptible to the chromatographic process on bonded silica packings. If ionic strength increased, electrostatic interaction will be weakened or screened in a certain region of ionic strength (0.1~ 0.3M in this study), resulting in the ideal size exclusion to dominate the retention mechanism.

The pH value of mobile phase determines the charge nature of a protein. It was shown that size exclusion dominates the retention mechanism in SEC for most of proteins at their isoelectric points. Ion adsorption could be induced if the pH value of mobile phase is lower than the isoelectric point of a protein.

The retention behavior of hemoglobin exhibited an obvious divergence from other proteins. It should be avoided in the use for detecting the separation capacity of a SEC column.

ACKNOWLEDGMENTS

This work was financially supported by the Natural Science Foundation of China and the Trans-century Excellent Talent Foundation of the State Education Commission of China to Z. He. The authors would like to thank the State Key Laboratory of Chemical Engineering at Tianjin University for providing them with HPLC and the PROTEIN PAK 300 sw column.

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Received July 22, 1997
Accepted August 9, 1997
Manuscript 4543